Isolation and characterization of a neomycin-resistant mutant of *Methanobacterium thermoautotrophicum* with a lesion in Na^+-translocating ATPase (synthase)

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Abstract A mutant of *Methanobacterium thermoautotrophicum* with a lesion in membrane Na^+-translocating ATPase (synthase) was isolated. The total ATPase activity in permeabilized cells of this mutant was elevated three-fold as compared with the wild-type strain. In contrast to wild-type cells, mutant ATPase was neither inhibited by DCCD nor stimulated by Na^+ ions. The methane formation orate of the mutant cells at pH 7.5 under non-growing conditions was nearly twice that of the wild-type strain and was stimulated by sodium ions. On the other hand, the ATP synthesis driven by methanogenesis under the same conditions was lower that of the wild-type under the same conditions, and contrary to the wild-type was not stimulated by Na^+ ions. ATP synthesis driven by a potassium diffusion potential in the presence of sodium ions was markedly diminished in the mutant cells. The membrane potential values of the wild-type and the mutant cells in the presence of 10 mM NaCl at pH 7.0 were comparable at energized conditions (−223 mV and −230 mV respectively). The Mg^{2+}-dependent ATPase activity of the $10^5 \times g$ supernatant of broken cells from the mutant cells was 30% higher than in the wild-type. On the other hand, two bands with Mg^{2+}-dependent ATPase activity were identified by native PAGE in this fraction in both wild-type as well as in mutant. These data suggest that the binding of Na^+-translocating ATPase (synthase) to the membrane spanning part is changed in the mutant strain.

Key words: Methanogen; Sodium ion-motive force; Na^+-translocating ATPase mutant

1. Introduction

A systematic genetic approach to the problem of energy conservation in yeast and eubacteria has proved to be very valuable [1–3]. In methanogenic archaea, a genetic approach to the solution of bioenergetic problems has not yet been applied. The problem of the co-existence of Na^+ and H^+ energetics has emerged lately in eubacteria [4–6]. Recent studies have revealed that the bioenergetics of methanogens exhibit unique features. (1) Methanogenesis is coupled to the generation of an electrochemical proton gradient and electrochemical sodium ion gradient [7–11]. (2) Both of these gradients are directly for ATP synthesis via specific H^+- and Na^+-ATP synthases [12–14]. To understand the function and interrelationship of these bioenergetic subsystems, functional (inhibitory) studies, physiological modifications and genetic elimination of the components of the bioenergetic machinery could be fruitful. Inhibitory studies and physiological modifications have shown that the cells of *Methanobacterium thermoautotrophicum* synthesize cellular ATP under alkaline conditions via Na^+-translocating ATP synthase [15]. This finding indicates that in cells of *Mb. thermoautotrophicum* ATP synthesis at different pH can be performed by one of two existing ATP synthases. Thus, the total energy expenditure of these cells may be met by either Na^+- or H^+-translocating ATPase (synthase).

The observation that Na^+-translocating ATPase (synthase) in *Mb. thermoautotrophicum* is inductive [16] could permit modulation of its activity at different physiological conditions. Moreover, this ATPase (synthase) seems to be F-type ATPase [12,13]. These experimental data, together with the possibility to prepare neomycin-resistant mutants in prokaryotes of which about 3% were found to have an uncoupled phenotype with defects in the energy-transducing ATPase [17,18], prompted us to try to prepare such mutants in *Mb. thermoautotrophicum*. The intention of this paper is to present the first case of a mutant of methanoarchaea with lesion(s) in its energy conservation mechanism.

2. Materials and methods

Mb. thermoautotrophicum strain ΔH was used as a wild-type strain and cultivated as described previously [19]. Growth medium was buffered to pH 7.5 with 50 mM morpholinopropane sulfonic acid (MOPS)-KOH. Solid media were prepared by the addition of 1% (w/v) Gelrite to the medium described above.

For isolation of neomycin-resistant mutants of *Mb. thermoautotrophicum* strain ΔH, the growth conditions were essentially as described above. To select neomycin-resistant mutants, cultures were grown for 24 h in liquid medium in the presence of 100 μg/ml neomycin. The cells were washed in the growth medium. 5×10^7 cells were plated directly in an anaerobic chamber (Forma Scientific, model 1024) on solid medium (pH 7.0) containing 100 μg/ml neomycin and were incubated at 60°C in an anaerobic jar for one week. Colonies that arose (42) were replica-plated on plates at pH 6.5 and 8.0 respectively with 100 μg/ml neomycin. After one week of growth, we compared the patterns of the corresponding plates. Only one mutant clone which grew at pH 6.5 and did not grow at pH 8.0 was isolated. This colony was picked and purified by restreaking several times on non-selective medium before the preparation of stock slopes. The remaining 98% of the mutant clones presumably carried lesions affecting the membrane permeability to neomycin. Specific growth rate (μ) was calculated from the exponential growth rate, absorbance range 0.2–0.8 at 580 nm.

ATP synthesis driven by a potassium diffusion potential in the presence of Na^+ ions of the cell suspensions of the wild-type and neomycin-resistant mutant was measured as described elsewhere [20].

For the study of ATPase activity permeabilized cells of *Mb. thermoautotrophicum* were used. Cells were permeabilized with Triton X-100 in a similar way as in [27].

Methane formation from CO_2 and H_2 by the cell suspension of the wild-type and neomycin-resistant mutant was measured by gas chromatography as described earlier [19]. Samples of the gaseous phase were taken from cultivation flasks (5 ml bottles sealed with butyl-

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rubber stoppers) with gas-tight syringes. The membrane potential was measured by means of an electrode sensitive to tetraphenylphosphonium (TPP^+) [21,22]. $10^5 \times g$ supernatant of broken cells was prepared as described by Roth and Bachofen [26] except that Tris buffer was replaced by *N*-(2-hydroxyethyl)piperazine-N′-3-propane-sulfone acid (HEPPS) buffer, containing 100 mM HEPPS-KOH, 10% glycerol (w/v), 10 mM $MgCl_2$ and 5 mM dithiothreitol (DTT), pH 8.0. The ultrasonification was performed in 20 s intervals on ice (20 min, 18 μm, Soniprep 150, R.W. Jennings and Co.). ATPase activity staining after native PAGE was performed according to Kakinuma and Igarashi [23]. Standard techniques were used for native PAGE.

Protein was quantified by the Lowry method with bovine serum albumin as the standard [24].

All chemicals were of reagent grade purity purchased mostly from Lachema, Brno, except for MOPS, HEPPS, Tris, ATP, DTT, dicyclohexylcarbodiimide (DCCD), Triton X-100, Gelrite (Serva), luciferin-luciferase preparation (Sigma), neomycin sulfate (Upjohn).

3. Results

Neomycin-resistant mutants of *Mb. thermoautotrophicum* were isolated. Only one of the 42 colonies was a small one, about one third in size, slightly yellowish and unlike the other resistants this one did not form visible colonies after plating on the alkaline solid medium at pH 8.0. These findings together with other reports on the isolation of ATPase mutants from neomycin-resistant mutants of bacteria [17,18] indicated that in our mutant a modification(s) in energy conservation mechanism might occur.

Growth yields (calculated as mg total cell protein per ml of growth medium after 24 h) were 0.22 for wild-type and 0.14 for the mutant strain. The specific growth rate μ (h^{-1}) in the wild-type cells at 5 mM NaCl was 0.05 and at 50 mM NaCl it was 0.15. In the mutant cells, the specific growth rate was the same (0.06) at both 5 mM or 50 mM NaCl.

Some neomycin-resistant strains of prokaryotic cells were found to have an uncoupled phenotype (unc) with defects in F-type ATPase [17,18]. Recently, it was suggested that cells of *Mb. thermoautotrophicum* strain ΔH contain the F-type as well as A-type ATPase (synthases) [13]. These findings prompted us to study relevant bioenergetic properties of this mutant to localize its defect(s).

Table 1 summarizes some basic features of Mg^{2+}-stimulated ATPase of permeabilized cells of wild-type and its neomycin-resistant mutant. In contrast to permeabilized wild-type cells, Mg^{2+}-stimulated ATPase activity of permeabilized mutant cells was neither stimulated by Na^+ nor inhibited by DCCD. Moreover, the basal activity was increased about three-fold compared with the wild-type. Similarly, Mg^{2+}-dependent ATPase activity in the $10^5 \times g$ supernatant of broken mutant cells was 30% higher than in this supernatant of wild-type cells (0.12 and 0.09 μmol of P_i/mg of protein/min, respectively). As described earlier [25,26], a large amount of ATPase activity was observed in such fractions of some thermophilic Archaea such as *Sulfolobus solfataricus*, *Mb. thermoautotrophicum* strain Hveragerdi and also *Mb. thermoautotrophicum* strain ΔH (unpublished results). Two bands with Mg^{2+}-dependent ATPase activity were detected after native PAGE in the $10^5 \times g$ supernatants of broken cells of both wild-type and mutant cells (data not shown). Relative mobilities of these bands were comparable to the mobilities of bands with ATPase activities obtained by analysis of EDTA extract of membrane vesicles of *Mb. thermoautotrophicum* strain ΔH [13].

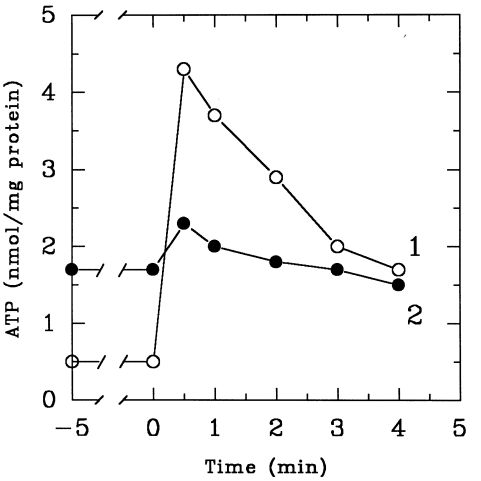

Fig. 1. Potassium diffusion potential-driven ATP synthesis in the wild-type and neomycin-resistant mutant. Cells were suspended in 50 mM MOPS-Tris buffer (pH 6.8) to a concentration of 1.8 mg protein/ml. ATP synthesis was induced by addition of valinomycin (15 μM final concentration) after 5 min of preincubation at 60°C. The NaCl concentration of the reaction mixture was 50 mM. 1, Wild-type; 2, mutant.

The properties of ATPase activity of mutant cells suggest a lesion in the Na^+-stimulated ATPase (synthase). Therefore we compared ATP synthesis driven by membrane potential which was generated either by artificially imposed ΔpH in the presence of uncoupler and Na^+ or valinomycin and Na^+ ions. In the former case the results obtained were ambiguous since the mutant cells agglutinated during the pH shift. In the latter case (Fig. 1) the ATP synthesis in mutant cells was considerably diminished. As shown in Table 2, mutant cells at pH 7.5 exhibited about two times higher production of methane than wild-type cells, in the presence of either 1 mm NaCl or 10 mM NaCl. In both cases the membrane production was stimulated by sodium ions. On the other hand, the ATP concentration in mutant cells was comparable with the concentration of ATP in the wild-type cells. The membrane potential values of the wild-type and the mutant cells at pH 7.0 and in the presence of 10 mM NaCl were identical, namely −233 mV and −230 mV (see Table 2).

Table 1
Effect of Na^+ and DCCD on ATPase activity (μmol of P_i/mg of protein/min) in premeabilized cells of *Methanobacterium thermoautotrophicum* and neomycin-resistant mutant

Addition	Wild-type			Mutant		
	Activity	Stimulation (%)	Inhibition (%)	Activity	Stimulation (%)	Inhibitition (%)
None	0.57	–	–	1.8	–	–
200 μM NaCl	0.43	–	24.6	1.78	–	5.6
15 mM NaCl	0.78	37.0	–	1.6	–	11.0
15 mM NaCl+200 μM DCCD	0.5	-	36.0	1.6	0	0

Table 2
Bioenergetic parameters of the wild-type strain and neomycin-resistant mutant with different Na^+ ion concentrations

Addition	Wild-type			Mutant		
	CH_4 (μmol/mg protein × 20 min)	ATP (nmol/mg protein)	Δψ (mV)	CH_4 (μmol/mg protein × 20 min)	ATP (nmol/mg protein)	Δψ (mV)
1 mM NaCl	7.00	3.10	−236	15.24	3.26	−226
10 mM NaCl	12.50	4.20	−233	24.20	4.40	−230

4. Discussion

The use of mutants in the study of bioenergetic problems in methanoarchaea may yield new information on this complicated system. In cells of many eukaryotes, prokaryotes and also in Archaea the problem of energy requirements is solved by two energy sources (electron transport phosphorylation and substrate-level phosphorylation). The total energy expenditure of these cells can be covered either by electron transport phosphorylation or by substrate-level phosphorylation depending upon the prevailing conditions. In methanoarchaea substrate-level phosphorylation seems to be absent. On the other hand, in methanoarchaea as in some eubacteria the co-existence of Na^+ and H^+ energetics was observed [12–15]. These data might indicate that methanoarchaea can utilize either both ATPases (synthases) or just one of them, depending on the energy of the corresponding gradients (Na^+ or H^+). Recently we have suggested that in *Mb. thermoautotrophicum* Na^+-translocating ATPase (synthase) might be the sole ATP synthesizing system when the proton-motive force-supported ATP synthesis via H^+-translocating ATP synthase is small (at alkaline pH) [15]. The use of mutants for the solution of this problem seemed to be very promising and attractive even if it had no precedent. The findings that in some methanogens there are two ATPases (A and F) cooperating with two coupling ions (Na^+ and H^+) [12,13] together with the possibility of preparing neomycin-resistant mutants in eubacteria with lesions in F_0F_1 [17,18] presented a challenge to prepare such mutants in methanoarchaea. Only one of the neomycin-resistant mutants was analyzed. Its size and growth characteristics indicated that this mutant might be the mutant of choice, the mutant with an unc phenotype. Since about 3% of the neomycin-resistant mutants in eubacteria have modifications in F-type ATPase, by analogy we expected that our mutant could have a similar phenotype. As F-type ATPase (synthase) is Na^+-translocating in *Mb. thermoautotrophicum* [13], we started to study the effect of sodium ions on ATPase activity of permeabilized cells. A 300% increase in the level of ATPase activity in the mutant strain but the absence of Na^+ stimulation and DCCD inhibition of this activity suggested some lesion(s) in the F_0 or F_1 portion of the ATPase of this mutant. Most of the ATPase mutants of prokaryotes have been shown to be affected in only one sector of the F_0F_1 complex, but some mutants of more complicated phenotype have been described that appear to be defective in both F_1 and F_0 [3]. The ATP synthesis driven by a potassium diffusion potential in the presence of sodium ions is also very low in mutant cells when compared with wild-type (up to five-fold lover). This result also indicates that this mutant has a deficiency in Na^+-dependent ATP synthesizing machinery. The finding that methanogenesis at pH 7.5 in the mutant cells is increased two-fold, while the rate of ATP synthesis driven by methanogenesis in wild-type is similar to that in mutant cells, indicates that ATP in the mutant cells is not formed by the Na^+-translocating ATPase because the sodium-motive force across the cytoplasmic membrane is dissipated. The membrane potential values of the wild-type and the mutant cells at pH 7.0 in the presence of sodium ions were identical. These data also support the idea that sodium ion-dependent energetics might be defective in mutant cells. The findings that mutant cells posses both H^+- and Na^+-translocating ATPases (synthases) together with the other experimental data strongly support the idea that in this mutant the binding of the ATPase catalytic moiety to the membrane spanning part at least for one ATPase (probably the Na^+-dependent one) is changed.

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References

[1] Kováč, L., Lachowicz, T.M. and Slonimski, P.P. (1967) Science 158, 1564–1567.
[2] Butlin, J.D., Cox, G.B. and Gibson, F. (1971) Biochem. J. 124, 75–81.
[3] Fillingame, R.H. (1981) Curr. Top. Bioenerg. 11, 35–106.
[4] Skulachev, V.P. (1994) Biochim. Biophys. Acta 1187, 216–221.
[5] Skulachev, V.P. (1994) J. Bioenerg. Biomembr. 26, 589–598.
[6] Lolkema, J.S., Speelmans, G. and Konings, W.N. (1994) Biochim. Biophys. Acta 1187, 211–215.
[7] Müller, V., Blaut, M. and Gottschalk, G. (1988) Eur. J. Biochem. 172, 601–606.
[8] Kaesler, B. and Schönheit, P. (1989) Eur. J. Biochem. 186, 309–316.
[9] Deppernmeier, U., Blaut, M., Mahlmann, A. and Gottschalk, G. (1990) Proc. Natl. Acad. Sci. USA 87, 9449–9453.
[10] Becher, B., Müller, V. and Gottschalk, G. (1992) FEMS Microbiol. Lett. 91, 239–244.
[11] Deppenmeier, U., Blaut, M. and Gottschalk, G. (1991) Arch. Microbiol. 155, 272–277.
[12] Becher, B. and Müller, V. (1994) J. Bacteriol. 176, 2543–2550.
[13] Šmigáň, P., Majerník, A., Polák, P., Hapala, I. and Greksák, M. (1995) FEBS Lett. 371, 119–122.
[14] Deppenmeier, U., Müller, V. and Gottschalk, G. (1996) Arch. Microbiol. 165, 149–193.
[15] Šmigáň, P., Majerník, A. and Greksák, M. (1994) FEBS Lett. 349, 424–428.
[16] Šmigáň, P., Horovská, L. and Greksák, M. (1988) FEBS Lett. 242, 85–88.
[17] Kanner, B.I. and Gutnick, D.L. (1972) J. Bacteriol. 111, 287–289.
[18] Sakai, Y., Moritani, Ch., Tsuda, M. and Tsuchiya, T. (1989) Biochim. Biophys. Acta 973, 450–456.
[19] Šmigáň, P., Friederová, A., Rusňák, P. and Greksák, M. (1984) Folia Microbiol. 29, 353–358.
[20] Schönheit, P. and Perski, H.J. (1983) FEMS Microbiol. Lett. 20, 263–267.
[21] Affolter, H. and Siegel, E. (1979) Anal. Biochem. 97, 315–319.
[22] Butsch, B.M. and Bachofen, R. (1984) Arch. Microbiol. 138, 293–298.
[23] Kakinuma, Y. and Igarashi, K. (1990) FEBS Lett. 271, 97–101.

[24] Lowry, O.H., Rosebrough, A.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[25] Hochstein, L.I. and Stan-Lotter, H. (1992) Arch. Biochem. Biophys. 295, 153–160.
[26] Roth, R. and Bachofen, R. (1994) Biochim. Biophys. Acta 1201, 271–283.
[27] Brandis, A., Thauer, R.K. and Stetter, K.O. (1981) Zbl. Bakt. Abt. Orig. C2, 311–317.